Retinoic Acid Regulation of Cdx1: an Indirect Mechanism for Retinoids and Vertebral Specification

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Retinoic acid (RA) is required for diverse developmental programs, including vertebral specification. Both RA receptor disruption and excess RA result in homeotic transformations of the axial skeleton. These effects are believed to occur through altered expression of *Hox* genes, several of which have been demonstrated to be direct RA targets. Members of the *cdx (caudal)* homeobox gene family are also implicated in regulating *Hox* expression. Disruption of *cdx1* results in vertebral homeotic transformations and alteration of *Hox* expression boundaries; similar homeosis is also observed in *cdx2* heterozygotes. In *Xenopus*, gain or loss of Cdx function affects vertebral morphogenesis through a mechanism that also correlates with altered *Hox* expression. Taken together with the finding of putative Cdx binding motifs in several *Hox* promoters, these data strongly support a role for Cdx members in direct regulation of expression of at least some *Hox* genes. Most retinoid-responsive *Hox* genes have not been demonstrated to be direct RA targets, suggesting that intermediaries are involved. Based on these findings, we hypothesized that one or more *cdx* members may transduce the effects of RA on *Hox* transcription. Consistent with this, we present evidence that *cdx1* is a direct RA target gene, suggesting an additional pathway for retinoid-dependent vertebral specification.

Retinoids such as retinoic acid (RA) play key roles in vertebrate development (43). The retinoid signal is transduced by two families of nuclear receptors: the RA receptors (RARs) and their isoforms (RARα1 and -α2; RARβ1, -β2, -β3, and -β4; and RARγ1 and -γ2) and the retinoid x receptors (RXRα, -β, and -γ). These receptors mediate ligand-dependent target gene transcription typically by binding as heterodimers to *cis*-acting RA response elements (RAREs) (6, 14, 29, 47).

Vertebral specification is believed to be governed by a Hox "code." This model is supported by a multitude of studies which demonstrate that anterior or posterior shifts in *Hox* expression or the ablation of specific *Hox* genes often leads to alterations in somite identity, as inferred by vertebral homeosis (10, 13, 31). Transplantation experiments suggest that vertebral specification, and hence *Hox* expression boundaries, is established in the unsegmented paraxial mesoderm at or shortly following gastrulation (36).

Both RAR knockout studies and studies on the effects of excess RA demonstrate roles for retinoids in vertebral morphogenesis (10, 31). RARγ null mice display axial malformations, including vertebral homeotic transformations (25). Although disruption of either RARα or RARβ2 does not affect skeletal development (27, 33), both receptors collaborate with RARγ in vertebral development, as judged by the marked increase in frequency and severity of axial skeletal defects in the corresponding double null mutants (26). A role for *Hox* genes in this program is suggested by the finding of altered expression of some *Hox* members following RA treatment in vivo. Moreover, certain *Hox* mutants are phenocopies of the axial transformations observed in RAR mutants (25, 26). However, despite these correlations, few RA-responsive *Hox* genes

have been shown to be direct RAR targets (11, 24, 35, 38, 39, 45).

Several lines of evidence suggest that vertebrate *caudal* homologues are key regulators of *Hox* expression. The murine *caudal* homologues *cdx1*, *cdx2*, and *cdx4*, are expressed in overlapping domains in the primitive streak region, with expression maintained in the posterior embryo through embryonic day 12.5 (E12.5) (5, 12, 34). These expression patterns suggest that a gradient of *cdx* function exists in the posterior embryo, which may reflect a means of regulating expression of different cohorts of *Hox* genes during somite specification (30). Consistent with this, *cdx1* null mutants as well as *cdx2* heterozygotes exhibit vertebral homeotic transformations (8, 46), which, in the former case, correlate with altered expression of certain *Hox* genes. The finding of consensus Cdx response elements in the promoter regions of several *Hox* loci (5, 46) further supports a role for Cdx members in direct regulation of *Hox* expression. Similar observations in *Xenopus* and *Caenorhabditis elegans* suggest that this pathway may be conserved (17, 20).

As most RA-responsive *Hox* genes are not known to be direct RAR targets, we hypothesized that a *cdx* member(s) may function as an intermediate. In support of this, we present evidence that *cdx1* responds to RA and RAR ablation in vivo in a manner consistent with it being a direct retinoid target. These results suggest an indirect pathway by which RA regulates *Hox* expression via direct control of *cdx1*.

MATERIALS AND METHODS

Animals. The RARγ, RARα1, and RARα1/γ null mice used in the present study have been described previously (26, 27). RARγ heterozygous and null embryos were generated from RARγ$^{+/−}$ intercrosses, whereas RARα1 and RARα1/γ null embryos were derived from RARα1$^{−/−}$γ$^{+/−}$ intercrosses. Wild-type embryos were obtained either from RARγ$^{+/−}$ matings, from intercrosses of wild-type stock from the RARγ colony (C57BL/6-129Sv hybrid), or from CD-1 intercrosses. No overt differences in gene expression or RA response were noted between any of these backgrounds. Females were dosed by oral gavage with all-*trans* RA dissolved in corn oil to a final delivery of 10 or 100 mg/kg of body weight at E7.5, E8.5, or E9.5 (noon of the day of plug appearance was considered

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E0.5). Animals were sacrificed 1 to 8 h posttreatment, and embryos were dissected in phosphate-buffered saline (PBS), fixed overnight in 4% paraformaldehyde, dehydrated through a methanol series, and stored at −20°C in 100% methanol. Yolk sacs were used to establish genotype by PCR as described previously (19). In some experiments, embryos were treated as described above and the presomitic caudal embryonic region was dissected out, snap frozen, and stored at −80°C prior to RNA isolation.

In situ hybridization analysis and embryo culture. Embryos were pooled by stage, genotype, and RA treatment and rehydrated. Whole-mount in situ hybridization was performed as described previously (50), using a riboprobe generated from the *cdx1* cDNA (34). After hybridization, embryos were cleared and photographed. Some specimens were then postfixed in 4% paraformaldehyde–0.2% glutaraldehyde at 4°C for 30 min, rinsed in several changes of PBS, embedded in Paraplast (Fisher), and sectioned.

Embryo culture was performed essentially as described previously (15). Embryos were dissected out in PBS containing 10% fetal bovine serum and stored briefly in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) buffered with HEPES. Embryos were cultured in DMEM–rat serum (50:50) preequilibrated with 5% O_2–5% CO_2 in N_2 at 37°C. Cultures were maintained for 4 h in the presence of RA (10^{-9} to 10^{-7} M in dimethyl sulfoxide [DMSO]) or vehicle (0.1%) prior to in situ hybridization analysis. In some experiments, cycloheximide (30 mg/ml) or the vehicle (EtOH, 0.1%) was also included in the culture medium for 30 min prior to addition of RA or DMSO. To monitor de novo protein synthesis in the latter experiments, 100 μCi of [^{35}S]methionine was added per ml, and incorporation of the label was assessed by filter binding as described previously (3).

Cell culture and transfection analysis. F9 embryocarcinoma cells were maintained in DMEM (Life Technologies) supplemented with glucose (4.5 g/liter), 10% fetal bovine serum, and gentamicin (10 μg/ml). For routine culture, cells were passaged every third day into gelatinized 100-mm tissue culture plates and cultured at 37°C in 5% CO_2. For Northern blot experiments, cells were seeded in 100-mm plates (approximately 10^6 cells/plate) and treated the following day with all-*trans* RA (1 μM) dissolved in DMSO (final concentration, 0.1%). Control cultures were treated with DMSO only. For Northern blot analysis, cells were harvested 2 to 48 h posttreatment, snap frozen, and stored at −80°C prior to RNA extraction.

To assess the requirement for de novo protein synthesis, cells were treated for 30 min with 15 or 30 μg of cycloheximide/ml or with the vehicle alone prior to RA treatment. Cells were then harvested, snap frozen, and stored as described above prior to Northern blot analysis. Parallel cultures were incubated with 40 μCi of [^{35}S]methionine/ml, and incorporation of the label was assessed by filter binding.

For transfection analysis, cells were passaged into gelatin-treated six-well cluster plates (approximately 10^5 cells/well) and transfected 24 h later using the calcium phosphate method. DNA mixtures were comprised of 1.5 μg of luciferase reporter construct, 0.75 μg of a *lacZ* expression vector as an internal control, and pBluescript KS(+), to a final concentration of 5 μg DNA per transfection. The following day, the medium was replenished and cells were treated with RA or DMSO, and culture was continued for 24 h. Monolayers were then rinsed twice in ice-cold PBS, and cells were disrupted by addition of 250 μl of lysis buffer (0.1 M Tris-Cl [pH 8], 1% NP-40, 1 μM dithiothreitol) for 5 min at room temperature. Cell lysates were collected and assessed for luciferase and β-galactosidase as described previously (3), and β-galactosidase activity was used to correct for transfection efficiency. Results were corrected for background (empty expression vector) and expressed as the means of three independent transfections. Unless otherwise stated, each experiment was repeated a minimum of three times.

To assess RA regulation in stable transfectants, 50 μg of the parental 2-kb *cdx1* reporter vector was linearized and cotransfected with 5 μg of a neomycin selection vector. Cells were selected by culture in the presence of 300 μg of G418 (Life Technologies)/ml for 2 weeks. Clones (approximately 100) were pooled and used to assess RA response by luciferase assay as described above.

Northern blotting and representative cDNA analysis. Total RNA was extracted from frozen embryos or cell pellets by using Trizol (Life Technologies) according to the manufacturer's directions. Fifteen micrograms of total RNA was resolved by electrophoresis through a formaldehyde gel and subjected to Northern blotting using Hybond N (Amersham) as described by the manufacturer. To quantify differences in embryonic gene expression, caudal tissue (posterior to the closed neural tube) was used for the generation of representative cDNA by PCR as previously described (18), followed by analysis by Southern blotting. Hybridizations were performed overnight at 42°C in a formamide-based buffer (40% formamide, 0.9 M sodium chloride, 50 mM sodium phosphate, 2 mM EDTA, 4× Denhardt's solution, 0.1% sodium dodecyl sulfate [SDS]) supplemented with 0.1 mg of denatured salmon sperm DNA/ml; denatured probe (approximately 10^6 cpm/ml) prepared by random priming was used. Blots were washed in 2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) three times at 65°C, followed by three washes in 0.2× SSC–0.1% SDS at the same temperature, and signal was revealed by autoradiography using X-Omat film (Kodak). For representative cDNA analysis, following autoradiography, densitometry was performed using Alpha Imager IS-1000 software (Alpha Innotech Corporation, San Leandro, Calif.). Values were normalized with respect to β-actin and expressed as fold change relative to untreated controls.

EMSA. The region of the *cdx1* promoter region conferring RA response, as defined by transfection analysis, was scanned by initially using fragments amplified by PCR. Each fragment was purified, end labeled with T4 polynucleotide kinase, and tested for RAR and RXR binding by electrophoretic mobility shift assay (EMSA). The putative RARE identified by this approach was evaluated for binding by EMSA with a double-stranded end-labeled oligonucleotide harboring either the sequence 5′-AAGGGTCGTGACCCT or the mutated sequence 5′-A AGGG<u>CAAGTT</u>CCCT (altered nucleotides are underlined). The end-labeled double-stranded oligonucleotide 5′-GGGGTAGGGTTCACCGAAAGTTCACT CGCA, harboring a consensus RARE (DR5), was used as a positive control in all binding assays. Nuclear extracts from Cos cells which had been either mock transfected or transfected with expression vectors encoding RARα and RXRγ were used as a source of protein. Binding reaction mixtures containing approximately 2 ng of probe (50,000 cpm) and 2 μl of nuclear extract (3 μg of protein) were equilibrated for 30 min at room temperature and separated by electrophoresis through a 5% polyacrylamide gel containing 0.25× Tris-borate-EDTA. For antibody supershifts, 0.5 μl of anti-RARα antibody (Santa Cruz) was added to protein extracts and equilibrated on ice for 30 min prior to addition of probe and further incubation as described above. Specificity of binding was assessed by competition with a 100-fold excess of unlabeled RARE nucleotides (sequences noted above) or with nucleotides harboring an SP-1 binding motif (5′-TCGAT CGGGGCGGGGCGA). In other EMSA experiments, electrophoresis was initiated at various times after probe addition or with various amounts of transfected Cos cell extracts.

Isolation of genomic sequences and derivation of plasmids. Sequences were isolated from a murine phage genomic library. A *Bam*HI-*Not*I fragment containing the endogenous transcription initiation site (16) and extending approximately 2 kb 5′ was ligated into the promoterless luciferase expression vector pXP2 (37), and subsequent deletion constructs were prepared by using convenient restriction sites. A reporter bearing the putative *cdx1* RARE was obtained by ligating either the double-stranded oligonucleotide 5′-AAGGGTCGTGACCCCT, harboring the wild-type sequences, or the mutated sequence 5′-AAGGGCAAGTT CCCCT into pTK109-Luc. A positive RA-responsive control, RARE-Luc, was derived by ligating the double-stranded oligonucleotide GGGGTAGGGTTC ACCGAAAGTTCACTCGCA, bearing the RARβ2 consensus RARE, into pTK109-Luc. Site-directed mutagenesis was performed using a Transformer kit (Clontech). All constructs were confirmed by sequencing.

RESULTS

RA induces *cdx1* in vivo. In untreated wild-type embryos at E8.5, *cdx1* transcripts were abundant in the ectoderm and mesoderm in the primitive streak region, with weaker expression in caudal neuroepithelium, in agreement with previous studies (Fig. 1A) (34). Eight hours following gavage with RA (100 mg/kg), expression was markedly increased (Fig. 1B) in all germ layers of the caudal embryo (Fig. 1D, compare to the control in 1C), with induction detectable as early as 1 h posttreatment (data not shown). Note that in this and other experiments, embryos to be compared were processed in parallel using the same probe to control for variables in signal intensity. Note also that experiments were terminated when strong staining was observed in any of the pooled samples. Therefore, untreated embryos were sometimes understained to clearly demonstrate the effect of RA.

In order to calculate induction, semiquantitative PCR was employed to compare *cdx1* transcript abundance in control and treated caudal embryo tissue. Treated embryos clearly exhibited a strong induction of message relative to untreated controls (Fig. 1E), whereas actin transcript abundance was not affected (Fig. 1F). Densitometric assessment of this regulation revealed a ninefold induction of *cdx1* signal when normalized for actin transcript abundance (Fig. 1G).

Embryo culture was employed to allow administration of known concentrations of RA and to more precisely regulate the time of exposure. In these experiments, both doses of RA tested (10^{-9} and 10^{-7} M) rapidly induced *cdx1* expression (Fig. 1I and J, compare to control in 1H). Notably, the lower concentration is in close agreement with the K_d of the RARs for RA (2), further supporting a physiological role for retinoids in regulating *cdx1* expression. Similar results were obtained by using a low dose of RA (10 mg/kg) in vivo at E8.5 (data not shown) and by using tissue culture models (see below).
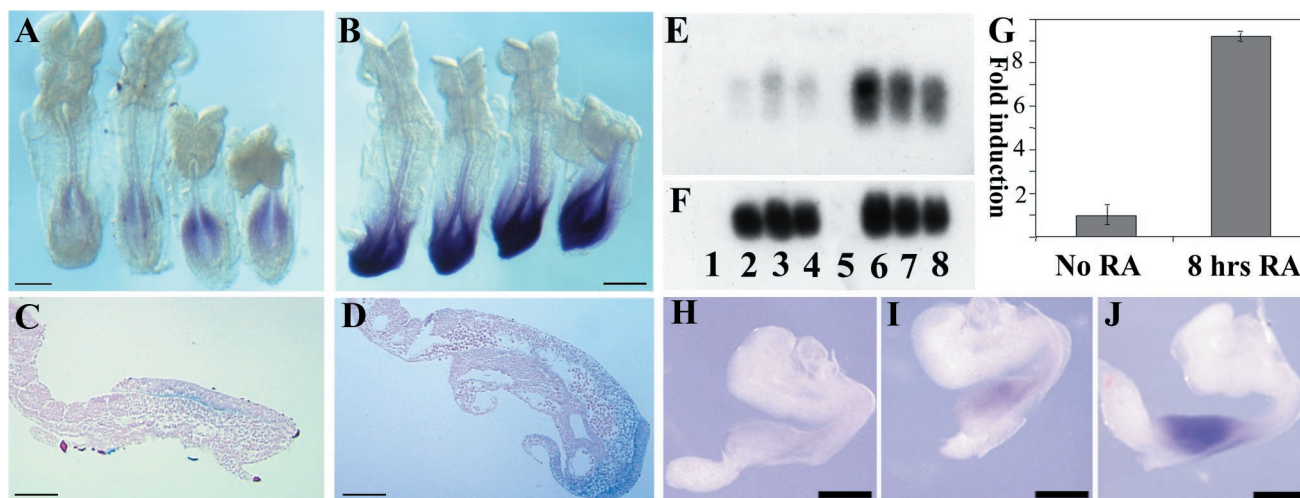
FIG. 1. Induction of *cdx1* by RA in vivo. (A and B) *cdx1* expression in E8.5 embryos treated with a vehicle (A) or 8 h following gavage with 100 mg of RA/kg (B). (C and D) Sagittal sections of embryos shown in panels A and B, respectively. Note the marked induction in signal throughout the caudal embryo. (E, F, and G) Semiquantitative analysis of *cdx1* expression. Representative cDNA Southern blots were generated from E8.5 caudal tissue, as described in Materials and Methods, and hybridized to either *cdx1* (E) or β-actin gene (F) probes. Compare basal expression (panel E, lanes 2 through 4) to expression following an RA treatment (lanes 6 through 8). β-Actin gene expression from the same material (F) was not affected. Densitometry from the blots shown in panels E and F was performed, and *cdx1* expression levels were normalized to β-actin gene expression levels. Results (G) are expressed relative to those for untreated controls and indicate a ninefold induction. Due to saturation of the X-ray film in the lanes from the treated samples, this is likely an underrepresentation of regulation. (H to J) Ex vivo response of *cdx1* to RA. E8.5 embryos were excised and cultured for 4 h in the presence of a vehicle (H), 10^{-9} M RA (I), or 10^{-7} M RA (J), following which *cdx1* expression was assessed by whole-mount in situ hybridization. Bar, 500 μm (A, B, H, I, and J) and 250 μm (C and D).

RA induces *cdx1* expression at several developmental stages. In the mouse, exogenous RA can induce vertebral homeosis from E7.5 to E9.5 in a manner that is coincident with altered *Hox* expression (22). We therefore determined if *cdx1* responded to RA throughout this window. In untreated embryos at E7.5, *cdx1* expression was observed in the ectoderm and mesoderm of the primitive streak region (Fig. 2A) as described previously (34), whereas RA elicited a strong induction of expression in the entire streak region at this stage (Fig. 2B). Interestingly, treatment appeared to induce *cdx1* precociously in embryos where expression either had not yet commenced or was only weakly detected (Fig. 2E, compare with F). At E9.5, *cdx1* transcripts in the caudal embryo were present at low levels (Fig. 2I). At this stage, hybridization was also observed in the forelimb bud mesenchyme, with weaker expression sometimes observed in the presumptive dermamyotome (Fig. 2I) (34). Four hours following treatment at E9.5, message was markedly induced in all of these domains, with a stronger signal consistently observed in the dermamyotome (Fig. 2J).

***cdx1* expression is altered in RAR null mutants.** *cdx1* expression was not overtly different in wild-type controls and RARα1 or RARγ single mutants at E7.5 to E9.5 (data not shown). In contrast, E7.5 RARα1/γ double mutants always exhibited reduced *cdx1* expression in the primitive streak region (Fig. 2C, compare with A). In marked contrast, transcript levels in the primitive streak region of these mutants at E8.5 were often comparable to levels in wild-type embryos (Fig. 2G, compare with 1A; also data not shown). At E9.5, expression in the tail bud was either comparable or too weak to compare between RARα1/γ mutants and controls. Similar variability was also seen with regard to expression in the limb buds and dermamyotome of these mutants, with expression sometimes weaker in the mutants than in stage-matched wild-type controls (Fig. 2K, compare with I). However, differences in expression were not consistently observed between RARα1/γ mutants and controls at E9.5. Similar variability in signal intensity was often seen in control E9.5 samples, suggesting

highly variable and dynamic expression of *cdx1* at this stage. Such variance precluded an accurate determination of the effects of RAR loss on *cdx1* levels in these embryos.

We also determined whether RARα1 and/or RARγ was required for induction of *cdx1* by exogenous RA. Following treatment at E8.5, caudal *cdx1* expression in RARα1 null embryos was comparable to expression in the wild type, whereas induction in RARγ mutants was only modestly reduced relative to that in the wild-type controls (data not shown). In contrast, induction in RARα1/γ mutants was markedly compromised in all normally responsive domains (primitive streak, dermamyotome, and forelimb bud) at all stages examined (compare untreated mutants in Fig. 2C, G, and K with the treated stage-matched samples in Fig. 2D, H, and L; also compare the relative induction in these double mutants to that seen in wild-type specimens at comparable stages (Fig. 2; see Fig. 1A and B for E8.5 wild-type embryos).

***cdx1* is a direct RA target.** To further investigate the effects of RA on *cdx1* expression, we employed F9 embryocarcinoma cells. In these cultures, RA up-regulated *cdx1* transcript levels as early as 2 h after treatment, with a maximum level attained after 24 to 48 h (Fig. 3A). In both F9 cells (Fig. 3B) and embryo cultures (Fig. 3C through F), this response was independent of de novo protein synthesis, as induction was evident in the absence or presence of cycloheximide (cycloheximide treatment resulted in ≥95% inhibition of de novo protein synthesis in either system). Notably, in embryo cultures, *cdx1* message was increased by cycloheximide treatment alone (Fig. 3E, compare to C), whereas a further increase in message abundance was seen upon subsequent treatment with RA (Fig. 3F). This superinduction effect suggests both an increase in transcription and a stabilization of message, a common phenomenon for immediate-early target genes.

We further investigated the mechanism of transcriptional regulation by using transfection approaches. The genomic region of *cdx1*, comprising approximately 2 kb of 5′ sequences, including the endogenous transcriptional start site and a por-
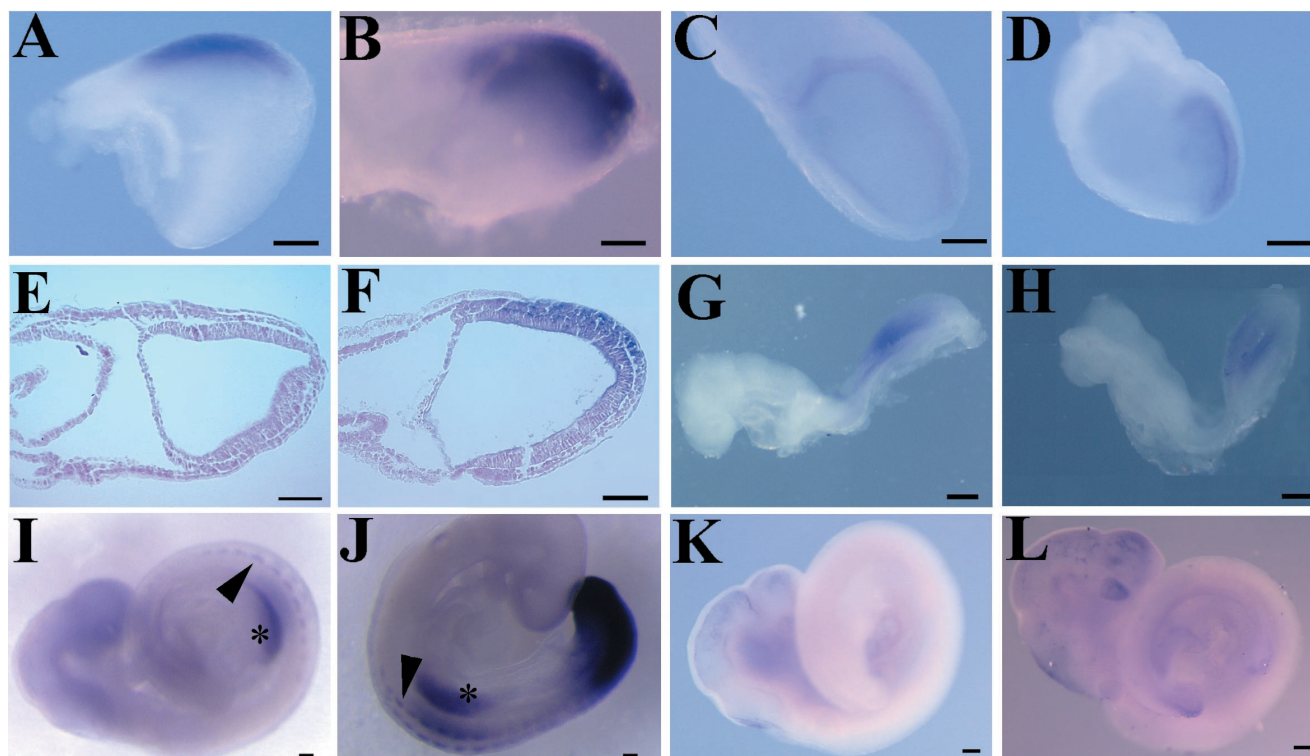
FIG. 2. *cdx1* expression in wild-type and RARα1/γ mutant embryos. Shown are results for whole-mount analysis of *cdx1* expression in E7.5 wild-type embryos without (A and E) or 4 h following (B and F) RA treatment. Note induction throughout the primitive streak region in the treated samples. (C and D) Expression in untreated (C) and RA-treated (D) E7.5 RARα1/γ mutant embryos. Note the reduced expression in the untreated mutant relative to the wild type (C versus A) and the decreased effects of RA on expression in the mutant background relative to controls (compare mutants in panels C and D to wild types in panels A and B). (I and J) *cdx1* expression in wild-type embryos at E9.5 without (I) and following (J) RA treatment. In untreated embryos, weak expression of *cdx1* was observed in the tail bud, with transcripts also evident in the forelimb bud and dermamyotome (asterisk and arrowhead, respectively, in panel I); RA strongly induced expression in all of these domains (J, compare to panel I). In RARα1/γ null embryos at E8.5 (G and H) and E9.5 (K and L), RA induction was reduced relative to that seen in the wild-type samples (compare effects of treatment in mutants in panels G and H and panels K and L to the effect on wild-type controls in Fig. 1A and B and Fig. 2I and J, respectively). Bars, 50 μm (A through F), 100 μm (G and H), and 75 μm (I through L).

tion of the 5′ untranslated region (5′-UTR) (16), was as potent as a synthetic RARE at eliciting an RA response in transient-transfection assays in F9 cells (Fig. 4A). Removal of sequences comprising the endogenous transcriptional start site and 5′-UTR revealed that the remaining, nontranscribed, sequences elicited an induction in transfection assays when coupled to a heterologous promoter (data not shown; see also below), suggesting that the RA response was not mediated through post-transcriptional mechanisms operating via the 5′-UTR. Finally, the 2-kb reporter was used to establish stably integrated reporter cell lines. These cell lines responded to low (10^{-9} M) levels of RA in dose-response experiments (data not shown), indicating that, as observed in vivo, this region conferred a response to physiological levels of RA.

Deletion analysis and transfection assays mapped the RA response region between −694 and −185 relative to the transcription start site (Fig. 4A). As a typical RARE (DR5) was not observed in these sequences, EMSAs were employed to identify the element. These experiments identified the motif AAGGGTCGTGACCCT as a target for RAR and RXR binding and demonstrated that all parameters of the *cdx1* RARE investigated by EMSA were identical to those exhibited by the control DR5 element (Fig. 4B, left and right sides, respectively). In particular, receptor binding to *cdx1* sequences was efficiently competed with excess unlabeled self or consensus DR5 RARE sequences, but not by an SP-1 binding motif or a mutated *cdx1* RARE. Conversely, the putative *cdx1* RARE, but

not the mutated element, competed efficiently for binding to the DR5 RARE. Specificity was further confirmed by super-shift assays, which demonstrated the presence of RARα in the complex. Although this supershift was not quantitative (for unknown reasons), the *cdx1* and DR5 elements exhibited identical degrees of antibody binding (Fig. 4B, compare supershift binding between lanes 4 and 11).

The relative affinity between the *cdx1* and DR5 motifs was also assessed either by varying the RAR-RXR concentration or by comparing relative binding as a function of reaction time. These data suggest that the *cdx1* motif is tightly associated with receptor complexes comparable to the DR5 control element, differing approximately twofold (Fig. 4D and data not shown). Moreover, the *cdx1* sequences exhibited only modestly slower kinetics of association relative to the DR5 element (Fig. 4E). These data are consistent with the finding that the *cdx1* RARE was absolutely essential for retinoid response in the context of the 2-kb promoter, as mutation of this motif completely abolished the response in F9 cells (Fig. 4C). Moreover, a single copy of this element was also sufficient to confer an RA response to a heterologous basal promoter (Fig. 4C). These *cdx1* sequences bear remarkable similarity to the rat growth hormone promoter TRE, the thyroid hormone response element, which has previously been shown to confer an RA response in transfection assays (49). The finding that this motif is perfectly conserved in the human *cdx1* promoter (data not shown) fur-
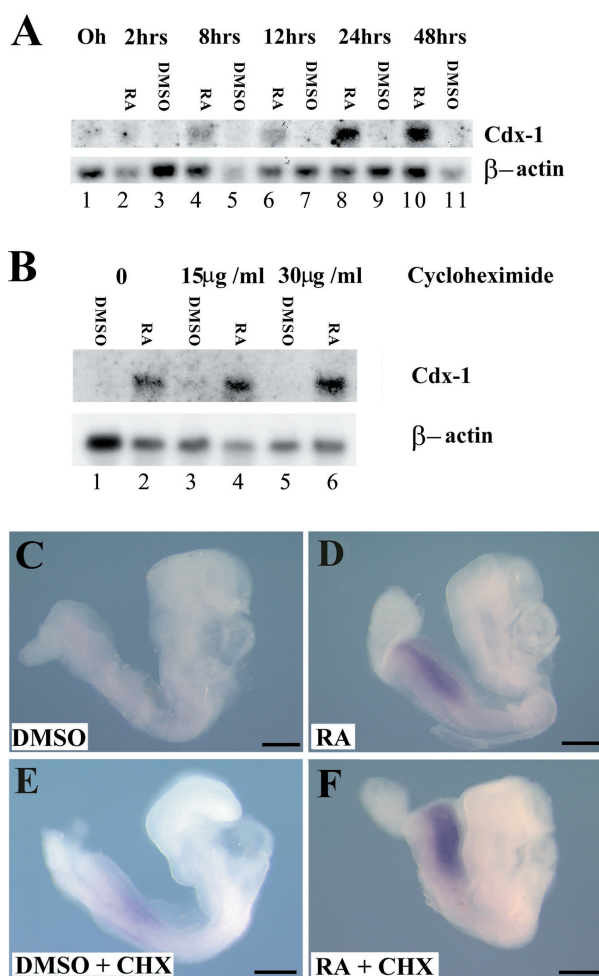
FIG. 3. *cdx1* induction is independent of de novo protein synthesis. (A) *cdx1* expression in F9 cells treated with RA (lanes 2, 4, 6, 8, and 10) or DMSO (lanes 3, 5, 7, 9, and 11) for the indicated times. (B) Northern blot of RNA from F9 cells treated for 4 h with RA (lanes 2, 4, and 6) or DMSO (lanes 1, 3, and 5) with the indicated amounts of cycloheximide. Both blots were reprobed for β-actin as a loading control. (C and D) *cdx1* expression in cultured embryos. Embryos were cultured for 4 h in the presence of a vehicle (C), 20 μg of cycloheximide (CHX)/ml (E), or 10^{-6} M RA (D) or RA plus cycloheximide (F) prior to in situ hybridization. Results shown are typical of several experiments. Bar, 400 μm (C to F).

ther suggests a conserved and important role for this element in directing expression.

DISCUSSION

Many *Hox* genes respond to RA both in tissue culture and in vivo, and this relationship is believed to be a principal means by which retinoids act in vertebral specification (10). Although a number of *Hox* genes have been shown to be direct RA targets, the mechanism(s) by which RA affects expression of most of the responsive *Hox* genes is largely unknown. Our present findings demonstrate that *cdx1* is a direct RA target, which, together with the established relationship between Cdx and *Hox* gene expression, strongly suggests a novel pathway for retinoids in axial specification.

Contribution of RA to *cdx1* expression. Findings from several models illustrate a role for *caudal* family members in anterior-posterior patterning. *cdx1* null mutants exhibit anterior vertebral homeosis which is coincident with posterior

shifts in the expression boundaries of certain *Hox* genes; similar vertebral defects are also observed in *cdx2* heterozygotes (8, 46). In *Xenopus*, gain or loss of the function of *Xcad* (the frog homologue of *cdx4*) results in patterning defects which correlate with altered *Hox* gene expression along the anterior-posterior axis (17). Taken together with the presence of potential Cdx response elements in a number of *Hox* promoters (7, 46), these data support a role for Cdx members in the direct control of *Hox* expression.

Our finding that *cdx1* is a direct RA target is in agreement with a number of observations. The homeotic transformations and rib fusions observed in *cdx1* null offspring (46) are reminiscent of the axial skeletal malformations exhibited by certain RAR null offspring (26). These similarities occur with respect to both the nature of the defects and their location along the vertebral column, being largely restricted to the cervical region in both classes of mutants. Consistent with this finding, a reduction in *cdx1* message was apparent in RARα1/γ mutants at E7.5. This is in agreement both with the window during which the cervical vertebrae are presumed to be specified and with the high frequency of vertebral defects observed in RARα1/γ mutants relative to RARα1 null offspring (which appear to be normal). However, reduction of *cdx1* expression was not observed in RARγ null embryos. This may relate to the low incidence of homeosis seen in these mutants (25), suggesting that effects on *cdx1* may be observed only at a correspondingly low frequency and/or may be too subtle to be readily detected by in situ hybridization techniques.

Our present data are entirely consistent with retinoid distribution studies. In the mouse, biologically active retinoids are first detected in the primitive streak region at E7.5 (4, 41). This correlates closely with the initial appearance of *cdx1* transcripts (34) and the ability of exogenous RA to precociously induce *cdx1* at this time. Moreover, *cdx1* expression was strongly reduced in RARα1/γ null embryos at E7.5. The finding that expression at E8.5 was not reproducibly altered in the double mutant background is likely related to the fact that retinoid activity is greatly reduced or absent in the primitive streak region commencing at this time (41). These data suggest that RA plays a role in the initial period of *cdx1* expression (perhaps to initiate expression) but that an additional factor(s) is involved in maintaining later phases of transcription. In this regard, in *Xenopus*, fibroblast growth factor (FGF) regulates *Hox* expression via control of *Xcad* (17, 40). Taken together with our present findings, this suggests that both retinoid signaling and FGF signaling converge on a common target gene. Indeed, FGF and RA act synergistically in inducing posterior *Hox* genes in *Xenopus* (9). However, whether this mechanism can be extrapolated to other vertebrates is unclear, as a relationship between FGF and *cdx* expression has not been described for the mouse.

RAR-specific regulation of *cdx1*. Studies with F9 cells suggest a key role for RARγ in induction of *cdx1* (48), an observation that contrasts with our findings in vivo. However, *cdx1* induction in F9 cells is only maximal after 24 to 48 h of treatment, in marked contrast to induction in vivo, which is evident 1 h posttreatment. This difference may be due, in part, to the fact that RARγ null F9 cells are refractory to RA-induced differentiation, suggesting that additional RARγ-dependent events impact *cdx1* transcription. Interestingly, another RA target gene, *CYP26*, exhibits similar differences between regulation in vivo and in F9 cells (1, 18). Although the basis for these discrepancies is speculative, these findings may be indicative of common cell-type-specific regulatory mechanisms which govern control of expression of these, and perhaps additional, RA target genes.
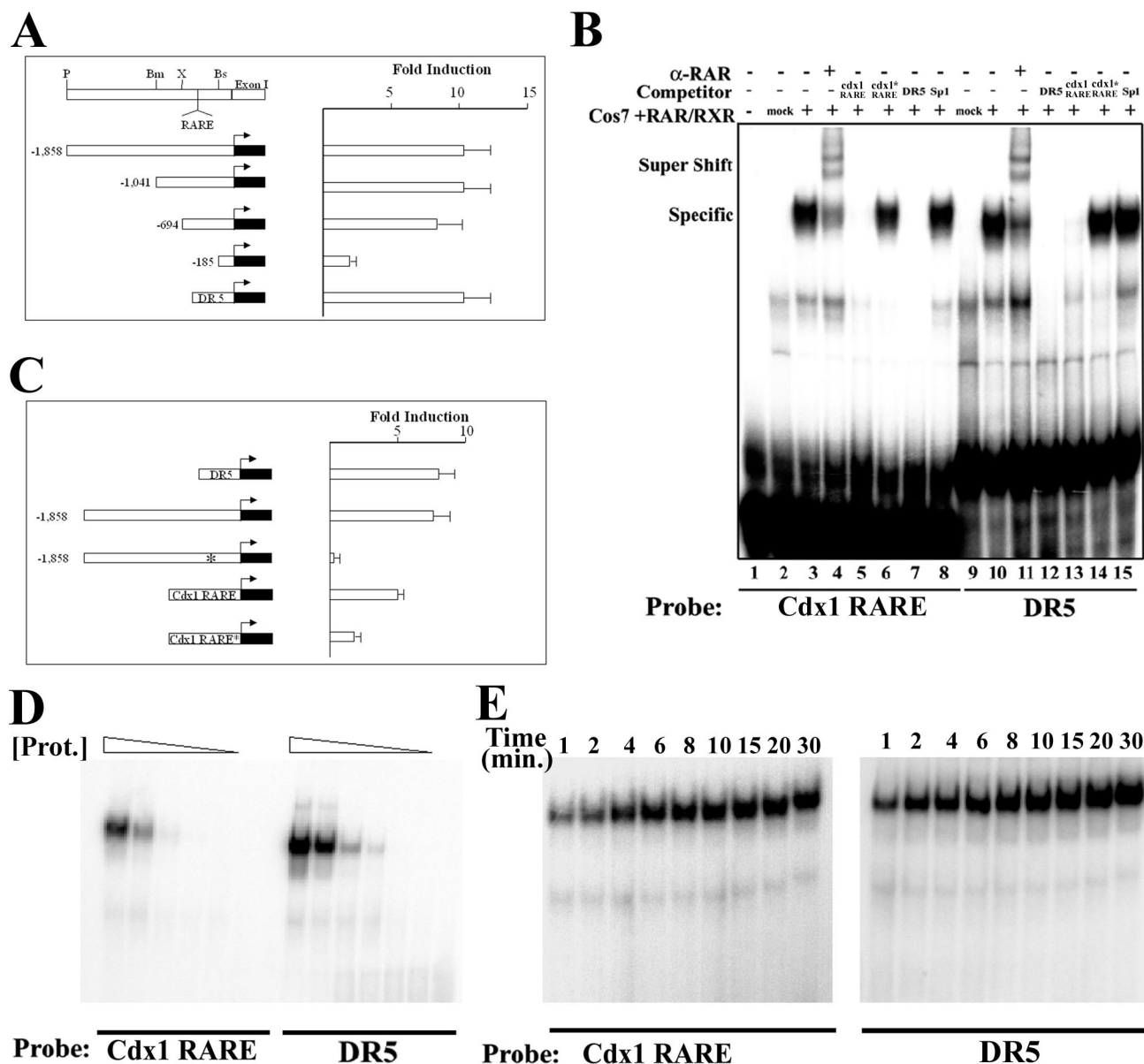
FIG. 4. Identification of an RARE in the *cdx1* promoter. (A) F9 cells were transfected with the indicated reporter constructs, and luciferase activity was determined 24 h following treatment with 10^{-6} M RA. Results are expressed as fold induction by RA relative to that in untreated cultures. Bm, *Bam*HI; Bs, *Bst*XI; P, *Pst*I; X, *Xmn*I. Numbering to the left indicates the 5′-most position relative to the transcriptional start site. DR5 is the positive control reporter construct. (B) The *cdx1* RARE motif was tested for receptor binding by EMSA using the radiolabeled oligonucleotide probes indicated at the bottom. Lane 1, no extract; lanes 2 and 9, mock-transfected cells; lanes 3 through 8 and 10 through 15, *cdx1* RARE and DR5 probe, respectively, incubated with extracts from cells transfected with RARα plus RXRγ. Specific binding complexes ("Specific") were not affected by nonspecific competitor (lanes 8 and 15) or by mutated *cdx1* RARE (lanes 6 and 14) but were competed by excess DR5 (lanes 7 and 12) or Cdx1 RARE (lanes 5 and 13). "Super Shift" indicates complexes formed by incubation with anti-RARα (lanes 4 and 11). (C) Transfection analysis, as in panel A, indicates that point mutation of the RARE sequences (∗) in the context of the 2-kb parental *cdx1* promoter results in complete loss of RA induction in F9 cells. A single copy of the *cdx1* RARE motif confers an RA response with a heterologous promoter, and this effect is lost upon mutation of these sequences (Cdx1 RARE∗). (D and E) Stability of the receptor association with *cdx1* RARE compared to the canonical DR5 RARE motif. (D) Comparable amounts of the probes were incubated with different amounts of extracts from receptor-transfected Cos cells for 30 min before electrophoresis. The protein (Prot.) amounts used were 3.00, 1.00, 0.30, 0.10, 0.03, 0.01, and 0.00 μg/incubation. (E) *cdx1* RARE and DR5 probes were incubated with 3 μg of Cos extract for the indicated amount of time, and binding complexes were resolved by electrophoresis.

RA, *Hox* expression, and somite specification. RA has been suggested to be a "posteriorizer" in the activation-transformation model of neurulation (reviewed in reference 42). RA can impart more posterior molecular characteristics on the anterior neuroepithelium, and interference with RAR signaling in *Xenopus*, or vitamin A deficiency in quail, results in hindbrain patterning defects, presumably due to effects on target genes such as *Hoxa-1* and *Hoxb-1* (11, 23, 28, 32, 44, 45). However, to date, a somite-specific RARE which is essential for expression of a *Hox* member with definitive function in paraxial mesoderm has not been described. As defects in *cdx1* null mice appear to be related only to somitic *Hox* misexpression, it is tempting to speculate that RA-dependent vertebral specification may manifest largely through *cdx1*. In contrast, the nonhomeotic axial

patterning defects observed in RARα1/γ double mutant offspring, which are not exhibited by *cdx1* mutants, clearly underscore the existence of other retinoid target genes involved in vertebral morphogenesis. The nature of these genes is unknown.

***cdx1* and retinoid-induced teratogenesis.** Excess RA has profound effects on vertebrate development and is capable of eliciting, among other malformations, neural tube and limb defects, axial truncation, and homeotic transformation, depending on both the dose and the embryonic stage upon exposure. As overexpression of *cdx* members can lead to neural tube defects in mouse embryos as well as caudal malformations of *Xenopus* tadpoles (7, 17), excess RA could conceivably exert some of its teratogenic effects through misexpression of *cdx1*. In this regard, RARγ is essential for retinoid-induced axial truncation (25). The finding that *cdx1* induction in RARγ mutants was only modestly compromised suggests that it is not involved in eliciting this malformation. However, in *Xenopus*, relatively small changes in *Xcad3* gene dosage result in dramatic differences in phenotypic outcome (17). Thus, we cannot exclude the possibility that the relatively small difference in *cdx1* induction in RARγ mutants is significant.

Current models suggest that up-regulation of *cdx* should lead to posterior transformation concomitant with anteriorization of *Hox* expression domains, whereas loss of Cdx function should lead to the converse situation. RA exposure at E7.5 to E9.5 induces *cdx1*, yet posteriorization events are seen only following treatment at E7.5. Exposure at later stages results in predominantly anterior transformation with loss of *Hox* expression (21, 22). At least two possibilities may explain these observations. First, as previously discussed (17), the overall level of Cdx proteins may be of importance, and induction of *cdx1* may be offset by reduction of *cdx2* (our preliminary observation) and *cdx4* (18), thus resulting in a loss of function at E8.5 and E9.5. Alternatively, Cdx members may differentially regulate target genes, as previously suggested (7). Additional studies are needed to address this as well as to further investigate the relationship between RA, *cdx*, and *Hox* expression.

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